Arginine-427 in the Na^+/glucose cotransporter (SGLT1) is involved in trafficking to the plasma membrane

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Abstract To investigate the role of charged intramembrane residues in the function of the rabbit Na^+/glucose cotransporter (rbSGLT1) we substituted arginine-427 (R427) by alanine in the putative domain M9 SGLT1. This residue is conserved in all the members of the SGLT1 family. The mutant protein (R427A) was expressed in *Xenopus* oocytes and, although Western blot analysis revealed that it was produced in amounts comparable to wild-type, no function was measured. Freeze-fracture analysis showed that R427A SGLT1 was not in the plasma membrane while immunocytochemical experiments localized the transporter to just beneath it. These results indicate that arginine-427 plays a critical role in SGLT1 trafficking to the plasma membrane.

Key words: Heterologous expression; Na^+/glucose cotransporter trafficking; Plasma membrane; Freeze-fracture; Immunocytochemistry

1. Introduction

An approach to structure/function relations of membrane proteins is site-directed mutagenesis, especially of conserved charged residues in transmembrane domains because they are thought to play an important role in protein function. For example, mutation of aspartic acid-176 to alanine (D176A) in transmembrane domain M4 of the rabbit Na^+/glucose cotransporter (rbSGLT1) alters the kinetics of charge transfer without changing the steady-state kinetics [1]. The interpretation of such experiments can be more complex because mutations can alter translation, processing and trafficking from the endoplasmic reticulum to the plasma membrane. In this study, we have changed arginine at position 427 of rbSGLT1 to alanine, expressed this protein in *Xenopus* oocytes and used a combination of techniques to address such questions. Although the protein was produced, no functional expression was obtained. This suggested a defect in either protein trafficking or in the function of the cotransporter. We then used freeze-fracture and immunocytochemistry methods to localize the mutated protein and found that R427A SGLT1 was not in the plasma membrane but it was just beneath it. We conclude that the substitution of a charged residue in the conserved transmembrane domain M9 of rbSGLT1 by a neutral amino acid (R427A) blocks the delivery of the transporter to the plasma membrane.

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2. Material and methods

2.1. Site-directed mutagenesis and cRNA synthesis

The plasmid carrying the cDNA sequence for rbSGLT1 [1] was used as template to perform oligonucleotide-directed mutagenesis [2]. The sequence of the synthetic oligonucleotide used to introduce the R427A mutation in its 5′→3′ direction was: CAGGAACAGCATGAACAAC-GCTCC TGCGATCATGA (mutated nucleotides in bold letters). The mutated fragment was ligated into the *Kpn*I/*Hin*cII sites of the wild-type vector and completely sequenced (Sequenase version 2.0, U.S.B., Cleveland, OH) to confirm the fidelity of the mutation. The recombinant plasmid then was linearized with *Eco*RI and in vitro transcription performed with a MEGAscript transcription kit from Ambion (Austin, TX).

2.2. Oocyte preparation and functional assays

Xenopus laevis oocytes were isolated, injected with cRNA coding for wild-type (WT) or R427A rbSGLT1 and 50 μM [^{14}C]-α-methyl-D-glucoside (αMDG) uptake was measured 3–7 days later [3]. SGLT1 membrane currents were measured using the two-microelectrode voltage clamp method [3].

2.3. Western blot analysis

Two oocytes expressing either wild-type or mutant rbSGLT1 were homogenized in 100 mM NaCl, 20 mM Tris-HCl, 1% Elugent (Calbiochem, La Jolla, CA), pH 7.5 (15 μl per oocyte) and centrifuged to remove the insoluble yolk and lipids. Protein samples were mixed with sample buffer, and a volume equivalent to 1/3 of oocyte was run on a 12% SDS-PAGE mini-gel and transferred to nitrocellulose membranes. Rabbit brush border membrane vesicles (BBMV) and non-injected (NI) oocytes were used as controls. SGLT1 was detected using polyclonal antibody raised to residues 602–613 of rb SGLT1 [4] at a dilution of 1:1,000. Affinity-purified goat anti-rabbit IgG peroxidase conjugate (Calbiochem, La Jolla, CA) at 1:12,000 dilution was used as secondary antibody. Immunoreactive proteins were visualized by chemiluminescence (Renaissance™ Dupont, Boston, MA).

2.4. Freeze-fracture and immunocytochemistry

Oocytes were fixed and freeze-fractured as previously described [5]. Oocytes expressing WT and R427A mutant proteins, non-injected oocytes and rat jejunum were fixed in 3% formaldehyde-PBS at 22°C for 2 h, rinsed 3 × 10 min with PBS and stored in 20% sucrose-PBS overnight at 4°C. Oocytes and tissue were embedded in OCT compound (Miles Inc., Elkhart, IN), rapidly frozen in dry ice and stored at −80°C. Cryostat sections of 6–8 μm thickness were cut, mounted on slides, dried and stored at −20°C. Sections were blocked for 10 min with 5% of normal goat serum-PBS and incubated for 1 h in a 1:100 dilution of an antibody raised to residues 564–575 of rbSGLT1 [6]. After washing with PBS 3 × 10 min, sections were covered with 10 μg/ml rhodamine-labeled affinity-purified goat anti-rabbit IgG (Jackson Immuno Research Laboratories Inc., West Grove, PA) for 40 min, and then washed with PBS 3 × 10 min. Intestinal sections were stained for nuclei with 2 μg/ml DAPI (4′,6-diamidino-2-phenylindole dihydrochloride, Research Organics Inc., Cleveland, OH). As controls, rat jejunum and oocytes expressing wild-type SGLT1 were incubated either in normal rabbit serum or PBS in the absence of primary antibody, and then with secondary antibody. Specimens were mounted in 200 mM DABCO (1,4-diazabicyclo[2.2.2]octane, Sigma Chemical Co., St Louis, MO), 90% glycerol, 10% PBS to retard fading of the fluorescence and exam-

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ined with a fluorescence microscope. Experiments with rat jejunum were carried out to confirm that the antibody recognized SGLT1 in the brush border membrane [6].

The sections were imaged by line averaging with a Zeiss LMS-10 laser confocal microscope (Carl Zeiss Inc., San Leandro, CA). Fluorescence was excited with the 567 nm band of an argon/krypton laser and the emission bandpass filter was 580–645 nm. The optical path included a X63 oil Plan Neofluar lens (numerical aperture 1.25). Contrast of the captured images was enhanced using Lynx 5.1 imaging software (Applied Imaging Co., Santa Clara, CA).

3. Results

The uptake of 50 μM [^{14}C]αMDG by the WT cRNA injected oocytes was about 100-fold higher than the uptake of R427A mutant (384 ± 36 and 3.7 ± 0.3 pmol/oocyte per h), which was not significantly different from non-injected oocytes (4.6 ± 0.4 pmol/oocyte per h). These results indicate that the R427A mutant does not transport αMDG or, if it does, with greatly reduced affinity. We tested this second possibility using electrophysiological methods. Fig. 1A shows the steady-state current-voltage relationship of the WT and R427A SGLT1 generated by 5 mM αMDG. At −150 mV membrane potential, WT produced a current of −2,230 nA whereas R427A did not induce an inward current at any membrane potential. In the absence of sugar, WT SGLT1 exhibits characteristic transient currents after voltage steps in the membrane potential. These currents are due to SGLT1 charge movement (Q), and Q_{max} is directly proportional to the number of transporters in the membrane [5,7]. Fig. 1B shows the charge movement of WT and R427A SGLT1 as a function of voltage. At each membrane potential (V), Q was determined as the integral of the transient currents (symbols) and the Q/V current was fitted to the Boltzmann equation [7]. Q_{max} for WT was 97 nC but for R427A was less than 7 nC.

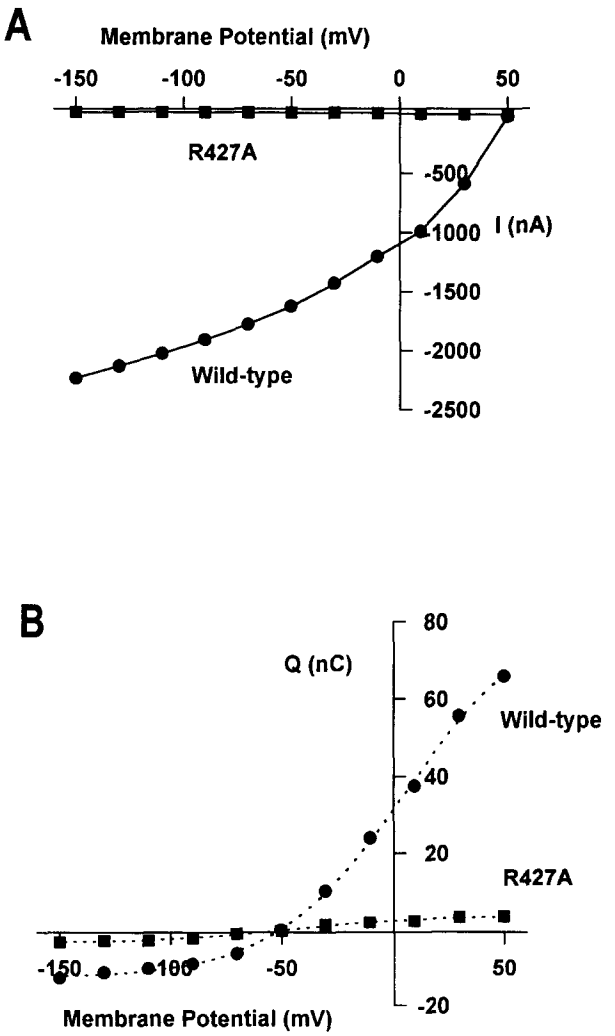

Fig. 1. Steady-state current-voltage (I/V) and charge-voltage (Q/V) relationship of WT and R427A rbSGLT1. (A) Current-voltage relationship of Na^+ inward current induced by 5 mM αMDG by WT and R427A SGLT1 proteins. (B) Voltage dependence of the charge movement. Q was obtained by integration of the current transients recorded in the absence of sugar at each membrane potential. The curves are drawn according to the Boltzman relation [7] and the symbols correspond to experimental data. The results are presented for single oocytes 6 days after injection of cRNA. Similar results were obtained in three different batches of oocytes.

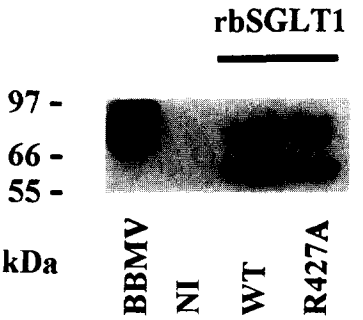

Fig. 2. Western blot analysis of wild-type and R427A mutant rbSGLT1 expressed in oocytes. Proteins were extracted from two oocytes 7 days after injection with WT or R427A mutant cRNA. The oocytes were from the same batch of oocytes used for Fig. 4. Lane 1, rabbit intestinal brush border membrane vesicles (BBMV) showing that the native glycosylated SGLT1 protein runs as a ~70 kDa band. Lane 2, non-injected oocyte (NI). Lane 3, wild-type rbSGLT1 expressed into oocytes which runs as two bands, one of the same size as the native intestinal brush border protein (lane 1) and a second, smaller band of ~60 kDa. Lane 4, R427A mutant which shows the same bands, at comparable intensities, as wild type.

The lack of functional expression of R427A SGLT1 could be due to defects in cRNA translation, trafficking of the synthesized transporter to the plasma membrane, or activity of the mutant protein. Western blot anlaysis was carried out to resolve if the cRNA was translated. Fig. 2 shows both WT and mutant proteins as two bands, one of the same size as BBMV (70 kDa) and a band of ~60 kDa that corresponds to the core-glycosylated protein.

The freeze-fracture method was then used to determine whether R427A protein was inserted into the plasma membrane. Fig. 3A shows the P face of an R427A cRNA injected oocyte where 7 nm diameter particles are found at a density of 190 ± 70 per μm^2. This is the same density as in water injected oocytes (212 ± 48 per μm^2) and corresponds to endogenous membrane proteins [5]. In WT cRNA injected oocytes the density of particles increased to ~5,000 per μm^2 (Fig. 3B) levels similar to that reported earlier for SGLT1 [5].

Immunocytochemistry experiments were performed to localize WT and mutant SGLT1 in the oocyte. Fig. 4 shows confocal images of WT (A) and R427A (B) SGLT1 expressing oocytes in which the background intracellular fluorescence (Fig. 4C and D) has been subtracted to highlight the differences in localiza-

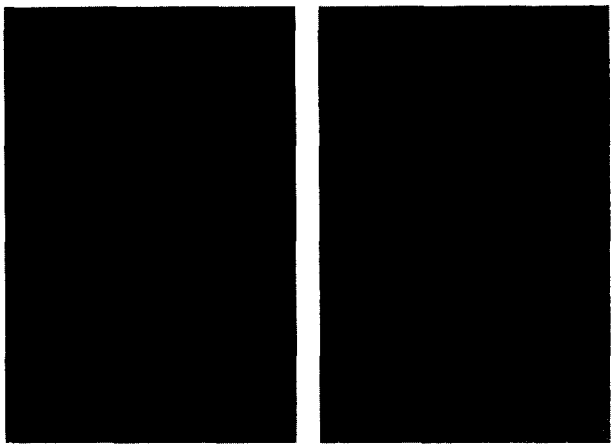

Fig. 3. The P fracture faces of plasma membranes of oocyte injected with R427A or wild-type cRNA. R427A (A) injected oocytes show 7 nm diameter particles at a density of 190±70 per μm^2, whereas in wild-type injected oocytes (B) the density of those particles increases to 5,000 per μm^2. There was no change in the density of E-face particles [5] Magnification: ×85,000.

tion of the two proteins. WT is restricted to the plasma membrane whereas R427A SGLT1 immunoreactivity appears just beneath it. No immunoreactivity was observed in either non-injected oocytes or oocytes not exposed to primary antibody.

4. Discussion

Site-directed mutagenesis is a popular tool to study the structure and function of cloned membrane proteins such as transporters, channels and receptors. However, as this study demonstrates, there is a serious limitation of this approach in expression systems when the mutant proteins are not inserted properly into the plasma membrane. We have found that simply changing a single residue in rbSGLT1 (R427A) dramatically alters the trafficking of the protein to the plasma membrane. Although there were comparable levels of WT and mutant proteins in the cell (Fig. 2), both sugar uptake and sugar-dependent currents for R427A SGLT1 were not significantly above control oocyte values (Fig. 1A). Charge movement measurements (Fig. 1B) and freeze-fracture electron micrographs of the plasma membrane (Fig. 3) further demonstrated that the level of the mutant protein in the plasma membrane was less than 7% of the WT. We therefore conclude that there was no functional expression of R427A SGLT1 in oocytes simply because the protein was not inserted into the plasma membrane.

In oocytes, as in other eukaryotes, membrane proteins are translated and assembled by the ribosome/endoplasmic reticulum (ER) complex, core glycosylated in the ER, and passed along the Golgi apparatus to the *trans*-Golgi where they are fully glycosylated. Then, plasma membrane proteins bud off from the Golgi apparatus in vesicles where they are directed to the plasma membrane. Western blot analysis of the mutant SGLT1 protein provides a clue about the site of the defect. The amounts of protein expression were similar for both mutant and wild-type transporters, as were the levels of core and complex glycosylation (Fig. 2). This suggests that the translation, insertion into the ER, folding, and trafficking of the R427A mutant SGLT1 to the *trans*-Golgi apparatus are normal. The immunocytochemical studies (Fig. 4) show that the mutant protein is found in a punctate pattern just below the plasma membrane. This, together with the glycosylation pattern and preliminary cell fractionation experiments (Hirayama, unpublished results), suggests that the R427A mutation blocks docking of the transport vesicle with the plasma membrane

How general is this effect of a mutation on trafficking?. A clue comes from our recent study of patients suffering from glucose-galactose malabsorption [8,9]. In 29 kindreds 18 different missense mutations were found in the SGLT1 gene that

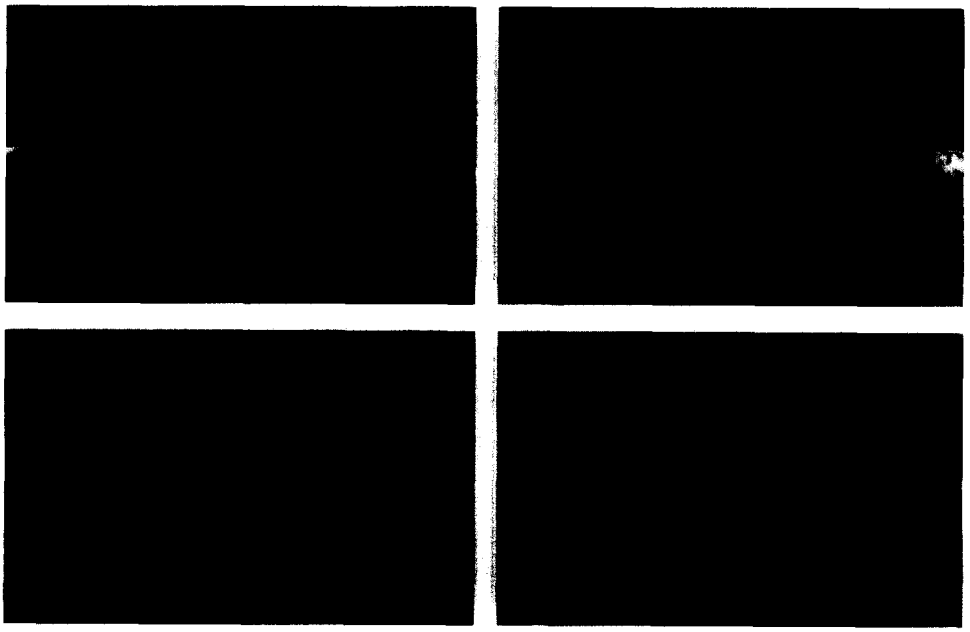

Fig. 4. Confocal fluorescence images (1 mm) of WT and R427A SGLT1 expressing oocytes. Panel A shows WT localized in the plasma membrane of the oocyte and panel B shows R427A localized beneath the membrane. Magnification: ×62. Panels C and D are the light microscopic semithin section (6–8 μm) from which the confocal images were taken. The oocytes were from the same batch as Fig. 2. Magnification: ×23.

account for the defect in sugar transport. Using the oocyte expression system, the missense mutations cause a severe impairment of sugar transport by SGLT1, and the defect appears to be mainly due to a trafficking problem: little or no charge transfer was observed with several mutants (D28N, A304V, R499H), and no D28N transporter was found in the plasma membrane using the freeze-fracture electron microscopic technique employed here (unpublished observations) even though Western blots showed normal levels of SGLT1 protein in the cells. In all of the GGM missense mutations and the R427A mutation studied here the mutated amino acid is highly conserved among the 12 members of the SGLT1 gene family [9]. Our results suggest that the trafficking and targeting machinery in oocytes interacts closely with the cotransporter protein in the transport vesicle, and that these interactions can be disrupted by changes in one out of 664 residues in the cotransporter sequence, including fairly conservative changes in amino acids, e.g. A3O4V and A388V.

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